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Intracellular calcium leak due to FKBP12.6 deficiency in mice facilitates the inducibility of atrial fibrillation

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Abstract

BACKGROUND—Although defective Ca^{2+} homeostasis may contribute to arrhythmogenesis in atrial fibrillation (AF), the underlying molecular mechanisms remain poorly understood. Studies in patients with AF revealed that impaired diastolic closure of SR Ca^{2+} -release channels (ryanodine receptors, RyR2) is associated with reduced levels of the RyR2-inhibitory subunit FKBP12.6.

OBJECTIVE—Objective of the present study was to test the hypothesis that Ca^{2+} leak from the sarcoplasmic reticulum (SR) through RyR2 increases the propensity for AF in FKBP12.6 deficient (−/−) mice.

METHODS—Surface ECG and intracardiac electrograms were recorded simultaneously in FKBP12.6−/− mice and wildtype (WT) littermates. Right atrial programmed stimulation was performed before and after injection of RyR2 antagonist tetracaine (0.5 mg/kg). Intracellular Ca^{2+} transients were recorded in atrial myocytes from FKBP12.6−/− and WT mice.

RESULTS—FKBP12.6−/− mice had structurally normal atria and unaltered expression of key Ca^{2+} -handling proteins. AF episodes were inducible in 81% of FKBP12.6−/−, but in only 7% of WT mice ($P < 0.05$), and were prevented by tetracaine in all FKBP12.6−/− mice. SR Ca^{2+} leak in FKBP12.6−/− myocytes was 53% larger than in WT myocytes, and FKBP12.6−/− myocytes exhibited increased incidence of spontaneous SR Ca^{2+} release events, which could be blocked by tetracaine.

CONCLUSIONS—The increased vulnerability to AF in FKBP12.6−/− mice substantiates the notion that defective SR Ca^{2+} release due to abnormal RyR2 and FKBP12.6 interactions may contribute to the initiation or maintenance of atrial fibrillation.

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Keywords

Atrial fibrillation; calcium; electrical remodeling; focal activity; ryanodine receptors

INTRODUCTION

The molecular mechanisms of atrial fibrillation (AF) are poorly understood. AF has been attributed to multiple re-entry circuits due to conduction and refractoriness abnormalities that are associated with profound alterations in the expression levels and posttranslational regulation of atrial ion channels^{1, 2}. However, emerging evidence points to the importance of focal mechanisms of arrhythmia in AF pathophysiology^{2, 3}.

Although the molecular mechanisms underlying ectopic focal activity in AF are unknown, it is accepted that dysfunctional Ca^{2+} homeostasis plays an important role⁴. Alterations in the subunit composition and posttranslational regulation of Ca^{2+} -handling proteins have been proposed as possible causes of abnormal Ca^{2+} handling^{4, 5}. Whereas the amplitude of the atrial L-type Ca^{2+} channel ($\text{I}_{\text{Ca,L}}$), the major trigger of Ca^{2+} -induced Ca^{2+} release from sarcoplasmic reticulum (SR), is reduced by ~60–70% in human AF^{6, 7}, there is substantial evidence for increased ryanodine receptor (SR Ca^{2+} -release channels; RyR2) activity in patients with AF^{5, 8}.

It was shown in patients with chronic AF that RyR2 channels exhibited increased serine 2808 phosphorylation and decreased binding levels of the inhibitory subunit FKBP12.6 (calstabin2)⁵. These RyR2 changes lead to an enhanced sensitivity to Ca^{2+} -induced activation at low cytosolic Ca^{2+} levels, which increases the propensity to spontaneous SR Ca^{2+} release events during diastole^{5, 9}. Enhanced SR Ca^{2+} -ATPase (SERCA2a) activity caused by increased phosphorylation of phospholamban (PLB) may further augment the likelihood of spontaneous SR Ca^{2+} releases in atrial myocytes from AF patients^{8, 10}. The resulting increases in cytosolic Ca^{2+} levels during diastole may activate an inward $\text{Na}^{+}/\text{Ca}^{2+}$ exchange current, which could cause delayed afterdepolarizations (DADs) and triggered activity promoting the genesis of atrial arrhythmias^{8, 11}.

The presence of concomitant cardiac diseases and use of medications complicate the interpretation of studies using atrial tissues from patients with AF^{5, 12}. To define the specific contribution of reduced FKBP12.6-binding to RyR2 in the development of diastolic Ca^{2+} leak associated with DADs and triggered activity, we performed intracardiac electrophysiology studies in a genetic mouse model of FKBP12.6 deficiency. Our studies reveal an increased vulnerability to AF in FKBP12.6^{-/-} mice, substantiating the notion that defective SR Ca^{2+} release may contribute to atrial arrhythmogenesis.

METHODS

FKBP12.6^{-/-} mice

All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine conforming the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). FKBP12.6-deficient (^{-/-}) mice and wildtype (WT) littermates, aged 3–5 months, were used for these studies.

Echocardiography

Mice were anesthetized using 1.5% isoflurane mixed with 95% O_2 and placed on a heated platform where all four limbs were taped to copper ECG electrodes. A rectal probe was inserted

for temperature monitoring. Apical four chamber views were recorded using a VisualSonics 35 MHz 707B probe (VisualSonics, Toronto, Canada).

Histology

Hearts from WT (n=6) and FKBP12.6^{-/-} (n=6) mice were perfusion-fixed with 4% buffered formaldehyde, embedded in paraffin, sectioned longitudinally (4–5 μ m), and stained with either hematoxylin-eosin (H&E) for cell morphology, or Masson trichrome for interstitial fibrosis using standard protocols.

Reverse transcription polymerase chain reaction and Western blot analyses

One μ g of RNA was used as template in a polymerase chain reaction after reverse transcription (rt-PCR) (SuperScript II, Invitrogen). Primers based on mouse cardiac LTCC (Cav1.2), NCX, RyR2, PLB, SERCA2a, and house keeping gene L7 cDNA sequences were employed to detect respective mRNAs. PCR products were resolved by 2% agarose gel electrophoresis.

Mouse atrial samples were prepared according to the method of Li *et al.*¹³. Western blotting was performed as described in the online supplement.⁹

Cardiac electrophysiology

Atrial and ventricular intracardiac electrograms were recorded using a 1.1F octapolar catheter (EPR-100, Millar Instruments) inserted via the right jugular vein. Bipolar right atrial pacing was performed using 2-ms current pulses delivered by an external stimulator (STG-3008, MultiChannel Systems). A computer-based data acquisition system (Emka Technologies) was used to record a 6-lead body surface ECG, and up to 4 intracardiac bipolar electrograms. Inducibility of AF was tested twice, according to the protocol described by Verheule *et al.*¹⁴.

After completion of all pacing protocols, 0.5 mg/kg tetracaine (20 μ l/min) or saline was infused into the left jugular vein of mice in which AF was induced following both burst pacing trials. Five minutes following tetracaine infusion, atrial burst pacing protocols were repeated (in duplicate) to determine inducibility of AF.

Telemetric ECG recordings

Mice were anesthetized using 2% isoflurane in O₂. ECG radiotelemetry recordings of ambulatory animals were obtained >48 hr following intraperitoneal implantation (Data Sciences International, St. Paul, MN)⁹. Standard criteria were used to measure ECG parameters¹⁵.

Calcium transient measurements

Atrial myocytes were isolated using standard enzymatic dissociation. Briefly, the heart was excised and immediately cannulated, attached to a Langendorff apparatus for retrograde perfusion, and digested using 0.1 mg/ml Liberase Blendzyme IV (Roche). Atria were dissected and tissue parts triturated to release single cells in Ca²⁺ free JMEM solution. Atrial myocytes were incubated with 5 μ mol/L Fluo-4 AM (Invitrogen) for 15 min at RT. Intracellular Ca²⁺ concentrations ([Ca²⁺]_i) were measured using an illumination device (model Lambda DG-4, Sutter Instruments), and an electro-multiplier intensified back-illuminated CCD camera (model Cascade 512B, Photometrics). Myocytes were preconditioned by pacing for at least 30 s before fluorescent signals were recorded at 1 Hz for 15 s, followed by a 15 s pause.

SR Ca²⁺ leak was measured as described in detail by Shannon *et al.*¹⁶. Briefly, [Ca²⁺]_i was measured in atrial myocytes using Fluo-4 AM as described above. Acute application of

tetracaine (5 $\mu\text{mol/L}$) was used to rapidly and reversibly block RyR2, thus disrupting the SR pump/leak balance. The tetracaine-dependent shift of Ca^{2+} from cytosol to SR (decrease in cytosolic $[\text{Ca}^{2+}]_i$ and increase in SR Ca^{2+} content) is proportional to SR Ca^{2+} leak in the absence of tetracaine¹⁶.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Student's *t* test and χ^2 test was applied when appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Normal atrial size and morphology in FKBP12.6 $^{-/-}$ mice

We used high-frequency echocardiography in apical four chamber views to determine atrial dimensions in FKBP12.6 $^{-/-}$ mice and WT littermates (Fig. 1A). There were no differences in right atrial systolic and diastolic dimensions between groups (Fig. 1B). Moreover, there was no evidence for cardiac hypertrophy (*data not shown*), as previously reported in a different strain of hypertensive FKBP12.6 $^{-/-}$ mice⁹. Following heart dissection, atria from FKBP12.6 $^{-/-}$ mice were macroscopically indistinguishable from WT with no differences in atrial weight-to-body weight ratios (1.0 ± 0.2 vs. 0.9 ± 0.1 mg/g wet weight; N.S.). Histological analysis of hematoxylin-eosin stained sections of FKBP12.6 $^{-/-}$ and WT mice revealed normal atrial size and microscopic structure (Fig. 1C, D). Masson trichrome staining demonstrated the absence of interstitial fibrosis in both groups (inserts in Fig. 1E, F). RT-PCR revealed unaltered mRNA levels of RyR2, Cav1.2, SERCA2a, PLB, and NCX in atria of FKBP12.6 $^{-/-}$ mice (Fig. 2A, C). Similarly, immunoblots showed unchanged protein levels of RyR2, Cav2.1, SERCA2a, PLB, and NCX in FKBP12.6 $^{-/-}$ atria (Fig. 2B, D). Furthermore, phosphorylation levels of the PKA phosphorylation site serine 2808 on RyR2 were similar in FKBP12.6 $^{-/-}$ and WT mice (Fig. 2B, D). Absence of FKBP12.6 mRNA and protein expression was confirmed in FKBP12.6 deficient mice. Finally, the phosphorylation status of serine 16 and threonine 17 on phospholamban was also unaltered in FKBP12.6 $^{-/-}$ mice (*data not shown*).

Baseline electrophysiological features of FKBP12.6 $^{-/-}$ mice

To determine the consequences of FKBP12.6 deficiency on baseline atrial electrophysiology, conventional 6-lead surface ECG and 4-lead bipolar intracardiac electrograms were simultaneously recorded in FKBP12.6 $^{-/-}$ and WT mice. There were no differences in heart rate, RR, PR, QRS, or corrected QT intervals on surface ECGs comparing FKBP12.6 $^{-/-}$ and WT mice (Fig. 3A, and Table 1). These findings were corroborated using bipolar intracardiac electrogram recordings (Fig. 3B–D). Endocardial programmed electrical stimulation was used to determine AERP, AVERP, and SNRT at 100 ms BCL, revealing no significant differences comparing FKBP12.6 $^{-/-}$ and WT mice (Table 1). During these electrophysiological studies, no episodes of spontaneous AF were observed in any of the FKBP12.6 $^{-/-}$ or WT mice. Moreover, telemetric ECG recordings revealed the absence of spontaneous atrial arrhythmias in 4 FKBP12.6 $^{-/-}$ mice monitored for 48–72 hours each (*data not shown*).

Pacing-induced AF episodes in FKBP12.6 $^{-/-}$ mice

To uncover a potential arrhythmogenic substrate in FKBP12.6 $^{-/-}$ mice, we performed a high-frequency atrial burst pacing protocol to provoke re-entry or atrial focal activity¹⁴. Inducibility of atrial arrhythmias was determined twice in each mouse using the stimulation protocol described by Verheule *et al.*¹⁴. Episodes of AF - defined as rapid and erratic atrial rhythm associated with irregular ventricular responses lasting > 1000 ms¹⁷ - were frequently observed in FKBP12.6 $^{-/-}$ mice (Fig. 4). Atrial arrhythmias were characterized by a high degree of beat-to-beat RR interval variability, with ventricular RR intervals ranging from approximately 60

to 130 ms. Episodes of pacing-induced AF were observed more frequently in FKBP12.6^{-/-} (81%, 21 of 26 mice) than in WT mice (7%, 1 of 14 mice) (Fig. 5). Mean duration of AF episodes in FKBP12.6^{-/-} mice, confirmed by rapid and irregular A waves on atrial electrograms, was 15.7 ± 3.5 s. We did not observe episodes of ventricular arrhythmias induced by right atrial pacing.

Tetracaine prevents pacing-induced AF episodes in FKBP12.6^{-/-} mice

To determine whether the increased AF susceptibility in FKBP12.6^{-/-} mice was mediated by enhanced SR Ca²⁺ leak through defective RyR2 channels, we repeated the burst pacing protocol following the administration of tetracaine. This local anesthetic drug has been shown to reduce the open probability of RyR2 channels in planar lipid bilayers¹⁸ and to decrease the frequency of spontaneous SR Ca²⁺-release events in rat ventricular myocytes¹⁹. We randomized the 13 FKBP12.6^{-/-} mice with reproducible pacing-induced AF to infusion with tetracaine (0.5 mg/kg i.v.; n=8) or placebo (saline; n=5). Five minutes following the infusion of tetracaine, baseline electrophysiological parameters were unaltered in both FKBP12.6^{-/-} and WT mice (Table 1). Neither AERP nor SNRT were affected by tetracaine (Table 1) excluding potential block of Na⁺ channels by the drug. Whereas placebo infusion did not prevent the occurrence of atrial arrhythmias in FKBP12.6 mice, tetracaine abolished the inducibility of AF in 100% (8 out of 8) of tested FKBP12.6^{-/-} mice (Fig. 5).

Enhanced diastolic SR Ca²⁺ leak in atrial myocytes from FKBP12.6^{-/-} mice

Although it has been shown that diastolic SR Ca²⁺ leak through RyR2 may trigger ventricular arrhythmias^{9, 20–22}, little is known about the potential link between SR Ca²⁺ leak and atrial arrhythmogenesis. Based on the higher incidence of pacing-induced atrial arrhythmias in FKBP12.6^{-/-} mice, we anticipated an increased diastolic SR Ca²⁺ leak in isolated FKBP12.6^{-/-} atrial myocytes. The SR Ca²⁺ leak, determined using a protocol described by Shannon *et al.*¹⁶, was estimated as the fractional decline in basal fluorescence in response to 5 μ mol/L tetracaine, and was related to the magnitude of the Ca²⁺-transient amplitude evoked by caffeine.

First, we determined average cross-sectional areas in atrial myocytes isolated from FKBP12.6^{-/-} ($1159 \pm 100 \mu\text{m}^2$; n=28) and WT ($1253 \pm 77 \mu\text{m}^2$; n=23) mice, to excluded atrial hypertrophy in the atria of FKBP12.6^{-/-} mice. During field stimulation, the amplitude of electrically evoked Ca²⁺ transients was comparable for FKBP12.6^{-/-} and WT mice (in arbitrary units [a.u.] of fluorescence: 2.61 ± 0.24 vs. 2.70 ± 0.44 , respectively). The time to 50% decay of Ca²⁺ transients was also similar in FKBP12.6^{-/-} (103.6 ± 12.8 ms) and WT mice (104.1 ± 6.6 ms).

However, the magnitude of SR Ca²⁺ leak was larger in FKBP12.6^{-/-} atrial myocytes (0.31 ± 0.04 a.u. of fluorescence) compared with WT cells (0.22 ± 0.04 a.u.; $P < 0.05$). Despite increased SR Ca²⁺ leak, steady-state SR Ca²⁺ content was similar in FKBP12.6^{-/-} myocytes (2.6 ± 0.2 a.u.) and WT mice (2.7 ± 0.4 a.u.). Thus, under conditions of comparable SR Ca²⁺ loads at resting conditions, SR Ca²⁺ leak was significantly higher in FKBP12.6^{-/-} (11.6 ± 0.8 %) compared with WT atrial myocytes (7.6 ± 0.9 %; $P < 0.05$; Fig. 6).

Increased frequency of spontaneous Ca²⁺-release events in atrial myocytes from FKBP12.6^{-/-} mice

We next evaluated whether enhanced SR Ca²⁺ leak was associated with an increased level of diastolic SR Ca²⁺ release events in FKBP12.6^{-/-} atrial myocytes. During the 10 sec immediately following termination of the pacing train, spontaneous SR Ca²⁺-release events were observed more frequently in myocytes from FKBP12.6^{-/-} (60%, n=60) compared with WT (9.4%, n=64) mice (Fig. 7). Whereas 35% of atrial myocytes from FKBP12.6^{-/-} mice

exhibited sub-threshold SR Ca^{2+} -release events, 32% generated full-size Ca^{2+} transients. In addition, an increased frequency of spontaneous Ca^{2+} releases was observed in FKBP12.6^{-/-} myocytes during the fast pacing train (*data not shown*). Tetracaine (5 $\mu\text{mol/L}$) reduced the incidence of spontaneous SR Ca^{2+} -release events in atrial myocytes from FKBP12.6^{-/-} mice to 11% (Fig. 7). Thus, FKBP12.6^{-/-} mice are more prone to spontaneous SR Ca^{2+} -release events, most of which are prevented by inhibition of RyR2 using tetracaine.

DISCUSSION

In the present study, we demonstrate that FKBP12.6 deficiency in mice is associated with an increased vulnerability to AF following atrial pacing. The increased susceptibility to AF is likely the result of spontaneous SR Ca^{2+} -release events due to leaky RyR2 channels in atrial myocytes of FKBP12.6^{-/-} mice. To the best of our knowledge, our study is the first to show specifically the consequences of increased SR Ca^{2+} leak for atrial arrhythmogenesis.

Abnormal intracellular Ca^{2+} release channel activity in AF

Recent studies have demonstrated that AF might not only be maintained by multiple re-entry circuits due to conduction and refractoriness abnormalities, but can also be sustained by focal activity due to triggered activity or abnormal automaticity^{3, 8}. Strong evidence has emerged that abnormal intracellular Ca^{2+} handling plays a central role in the pathogenesis of focal sources and triggered activity in AF^{5, 8, 10}. Defects in sarcolemmal ion channels, in particular decreased $\text{I}_{\text{Ca,L}}$ current, underlie electrical remodeling in AF, but are not sufficient to explain the enhancement of spontaneous Ca^{2+} -release events in myocytes from AF patients^{6, 7}.

Atrial myocytes from patients with paroxysmal and chronic AF exhibit more frequently spontaneous SR Ca^{2+} -release events (Ca^{2+} sparks) than myocytes from patients in sinus rhythm despite downregulation of the $\text{I}_{\text{Ca,L}}$ current⁸. The increased frequency of potentially arrhythmogenic Ca^{2+} sparks and Ca^{2+} waves are most likely caused by defective RyR2 channels^{5, 8, 10}. Consistent with these observations, we previously found defects in RyR2 in atrial biopsies of patients in chronic AF. In particular, both increased PKA phosphorylation of serine 2808 on RyR2 and decreased binding of the channel-stabilizing FKBP12.6 subunit (calstabin2) were observed⁵. Similar channel defects were observed in a canine model of sustained AF with preserved ventricular function, suggesting that these defects are not secondary to altered ventricular contractility⁵.

Atrial arrhythmias associated with FKBP12.6 deficiency

To determine the specific role of reduced FKBP12.6 binding to RyR2 in AF pathophysiology, we studied atrial susceptibility to pacing-induced AF in FKBP12.6-deficient mice. We demonstrate, for the first time, that SR Ca^{2+} leak is larger in atrial myocytes from FKBP12.6^{-/-} mice and underlies the increased incidence of spontaneous Ca^{2+} -release events and the higher vulnerability to inducible AF episodes. Lehnart et al.²³ recently demonstrated that spontaneous SR Ca^{2+} releases in ventricular myocytes from FKBP12.6^{-/-} mice cause delayed afterdepolarizations (DADs), suggesting that SR Ca^{2+} leak can also trigger arrhythmogenic DADs in atrial myocytes. However, due to technical difficulties of isolating mouse atrial myocytes and conducting such measurements, we were unable to directly confirm this in the present study.

A larger SR Ca^{2+} load is probably not the primary trigger of SR Ca^{2+} leak because neither amplitude nor time to 50% decay of the Ca^{2+} transients nor SR Ca^{2+} load (as estimated with caffeine) differ between FKBP12.6^{-/-} and WT myocytes. On the other hand, enhanced SR Ca^{2+} leak and vulnerability to AF in FKBP12.6^{-/-} mice is most likely mediated by leaky RyR2, as inhibition of RyR2 by tetracaine suppressed both phenomena. The preserved protein

levels of RyR2, Cav1.2, SERCA2, and PLB in atria of FKBP12.6^{-/-} mice also suggest that the observed atrial arrhythmogenicity in FKBP12.6^{-/-} atria most probably results from abnormal RyR2 function due to the altered subunit composition of the channel complex. Potential changes in phosphorylation of RyR2 are unlikely to contribute to increased SR Ca²⁺ leak in FKBP12.6^{-/-} myocytes because the phosphorylation levels of serine 2808 on RyR2 were similar in atria of WT and FKBP12.6^{-/-} mice. Moreover, the absence of fibrosis in the atria of FKBP12.6^{-/-} mice also suggests that the initiating trigger for AF results from abnormal Ca²⁺ release within atrial myocytes, as supposed to reentry due to remodeling of the extracellular matrix.

Mechanisms of atrial arrhythmias in FKBP12.6^{-/-} mice

Although the lack of FKBP12.6 confers increased susceptibility to pacing-induced AF, FKBP12.6 deficiency by itself does not appear to promote the development of spontaneous AF, suggesting that the absence of FKBP12.6 alone does not cause enough SR Ca²⁺ leak to trigger AF. Enhanced Ca²⁺ loading of the SR during atrial tachycardia in combination with increased 'leakiness' of RyR2 in the absence of inhibitory protein FKBP12.6 appears to be a prerequisite for AF inducibility. Thus, the sarcoplasmic reticulum in FKBP12.6^{-/-} atrial myocytes releases Ca²⁺ spontaneously through FKBP12.6-deficient RyR2 whereas wildtype myocytes have the ability to retain Ca²⁺ inside the SR during diastole despite increased Ca²⁺ filling during pacing⁹. Translating these findings to the whole organ level, it is likely that Ca²⁺-mediated triggered activity and abnormal Ca²⁺-cycling dynamics facilitate re-entry by RyR2-mediated Ca²⁺-dependent alternans and associated wavebreak, as proposed by Weiss *et al.*²⁴. Whereas the amplitude of the increased SR Ca²⁺ leak in FKBP12.6^{-/-} myocytes appears to be sufficient to induce atrial arrhythmias, the enhanced SR leak did not significantly affect SR Ca²⁺ loading under our experimental conditions in isolated atrial myocytes. Since similar findings were made in human atrial myocytes (i.e., increased SR Ca²⁺ leak despite unaltered SR Ca²⁺ loading), increased RyR2 leakiness may indeed play a critical role in atrial arrhythmogenesis of patients with AF.

Study limitations

Due to the very small sample sizes, we were not able to directly measure the open probability of RyR2 from mouse atria using the planar lipid bilayer assay. However, the arrhythmogenic effects of FKBP12.6 deficiency were almost completely reversed by tetracaine, an inhibitor of RyR2 channels. Tetracaine also unmasked larger SR Ca²⁺ leak in FKBP12.6 deficient mice indirectly indicating enhanced open probability of RyR2 channels. However, we could not exclude the possibility that tetracaine suppresses re-entry circuits by blocking fast sodium currents in the atrium.

One limitation of our mouse electrophysiology model is that induced arrhythmias during overdrive pacing may be dissimilar from the mechanisms occurring during spontaneous cardiac arrhythmias in humans. Nevertheless, it has been reported that the mouse atria are able to sustain fibrillation^{14, 17, 25, 26}. Thus, despite the small sizes of mouse atria, our knockout mouse allowed for the investigation of the mechanisms underlying the genesis and maintenance of AF. Unlike in larger animals or patients, it is presently not feasible to explore possible regional differences in fibrillation properties using catheter-based EP studies. Future studies involving optimal mapping techniques may provide further insight into the electrophysiological mechanisms contributing to atrial arrhythmias caused by FKBP12.6 deficiency.

Finally, it is possible that the effects of FKBP12.6 deficiency in human atria and especially in diseased hearts are different than those in mice and of lesser importance in promoting an arrhythmogenic substrate for AF maintenance. In episodes of AF that occur through the

transition from supraventricular tachycardia to AF, increased SR Ca^{2+} loading and enhanced SR Ca^{2+} leak might be the primary arrhythmogenic mechanism. However, in patients who develop AF *de novo* without preceding atrial tachycardia, this mechanism may not contribute to the initiating arrhythmogenic beat.

CONCLUSION

Our findings show that aberrant SR Ca^{2+} release via FKBP12.6-depleted RyR2 channels may be one of the mechanisms promoting the ability of AF to sustain in structurally normal atria with increased vulnerability to atrial arrhythmias. The reduction of SR Ca^{2+} leak via defective RyR2 channels may constitute a novel strategy to reduce atrial arrhythmogenesis^{20, 27, 28}. Additional studies in larger mammals and patients with AF are needed to confirm the therapeutic potential of RyR2-based therapies for AF.

CLINICAL IMPLICATIONS

Clinical atrial re-entry is rarely induced by a single premature beat. In most cases, re-entry induction requires bursts of rapid atrial activation. Spontaneous SR Ca^{2+} releases associated with the potential generation of DADs and triggered activity in the absence of FKBP12.6 are strong candidates to fulfill this function. Ca^{2+} -dependent triggered activity could contribute to AF genesis by either producing atrial tachycardia bursts that trigger re-entry in the presence of vulnerable substrates or by providing focal sources that drive AF. Since FKBP12.6 binding to RyR2 is reduced by about 50% in patients with chronic AF⁵, diastolic Ca^{2+} leak through FKBP12.6-depleted RyR2 Ca^{2+} channels may contribute to both promotion of induction and maintenance of AF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. FKBP12.6-deficient mice have structurally normal atria

(A) Representative echocardiographic image showing apical four-chamber view of an FKBP12.6^{-/-} mouse heart. Arrow indicates right atrium. White scale bar (top) represents 2 mm. (B) Right atrial systolic and diastolic areas were similar in WT and FKBP12.6^{-/-} mice. (C, D) Hematoxylin-eosin stained sections of WT (C) and FKBP12.6^{-/-} (D) mouse hearts. Scale bar, 2 mm. (E, F) Masson Trichrome-stained sections reveal absence of fibrosis in FKBP12.6^{-/-} atria. Scale bar, 20 μ m.

Fig. 2. Expression of major Ca^{2+} -handling proteins in FKBP12.6 $^{-/-}$ atria

(A) rt-PCR and (B) Western blotting demonstrate unaltered (A) mRNA and (B) protein levels of Ca^{2+} -handling proteins in FKBP12.6 $^{-/-}$ mice compared with WT mice. Bar graphs show normalized (C) mRNA and (D) protein levels. Numbers indicate mice tested.

Fig. 3. Normal baseline surface ECG and intracardiac electrograms in FKBP12.6^{-/-} mouse (A) Representative lead II surface ECG in WT (*left*) and FKBP12.6^{-/-} mice (*right*). Intracardiac recordings from WT (*left*) and FKBP12.6^{-/-} (*right*) mice show atrial (B), atrio-ventricular (C), and ventricular (D) electrograms. A, atrial depolarization; V, ventricular depolarization; P, P wave; R, R wave; Q, Q wave.

Fig. 4. Rapid pacing-induced atrial fibrillation in FKBP12.6^{-/-} mouse

(A) Lead II surface ECG recording showing right atrial pacing-induced AF in FKBP12.6^{-/-} mouse with beat-to-beat variability in RR interval times. (B) Intracardiac electrogram with disorganized fibrillatory activity in the right atrium. (C) Right atrial/ventricular and (D) right ventricular electrograms showing irregular ventricular depolarizations. (E) Right atrial burst output (see arrow). A, atrial depolarization; V, ventricular depolarization.

Fig. 5. Tetracaine prevents atrial fibrillation in FKBP12.6-/- mice

Bar graphs showing the incidence of AF in WT (n=14) and FKBP12.6-/- (n=26) mice. The black bars indicate the percentage of mice in which AF was induced following 2 out of 2 of burst pacing trials. The grey bars indicate the percentage of mice in which AF was induced once during the 2 pacing trials.

Fig. 6. Increased SR Ca^{2+} leak in atrial myocytes from FKBP12.6^{-/-} mice

Representative $[Ca^{2+}]_i$ recordings obtained from fluo-4 AM-loaded atrial myocytes from WT (A) and FKBP12.6^{-/-} (B) mice during 1 Hz pacing in 1.8 mM Ca^{2+} (left), followed by a rapid switch to Tyrode solution containing 0 Na⁺, 0 Ca^{2+} , and tetracaine (1 mmol/L). Tetracaine is washed out 30 sec later, and SR Ca^{2+} content is measured by changing superfusate to 10 mmol/L caffeine in 0 Na⁺, 0 Ca^{2+} Tyrode. SR Ca^{2+} leak was measured as shown in red, and related to SR Ca^{2+} content. (C) Bar graph showing increased SR Ca^{2+} leak in FKBP12.6^{-/-} atrial myocytes. * P<0.05.

Fig. 7. Increased incidence of non-evoked Ca²⁺ oscillations in FKBP12.6^{-/-} atrial myocytes
 Representative fluorescence recordings obtained from fluo-4 AM-loaded single atrial myocytes from WT (A) and FKBP12.6^{-/-} (B) mice subjected to the leak protocol. Please note spontaneous sub-threshold Ca²⁺ releases (arrow heads) and non-evoked Ca²⁺ transient (*) in the FKBP12.6^{-/-} myocyte. (C) Tetracaine (5 μ mol/L) inhibited spontaneous Ca²⁺ oscillations in FKBP12.6^{-/-} myocytes, see bar graph (D). * P < 0.05.

Table 1Normal cardiac electrophysiological parameters in WT and FKBP12.6^{-/-} mice.

Analysis	Baseline		Post Tetracaine	
	WT (n=14)	FKBP12.6 ^{-/-} (n=26)	WT (n=7)	FKBP12.6 ^{-/-} (n=8)
RR, ms	115.4±2.4	119.6±3.0	109.2±2.3	112.6±2.5
HR, bpm	522.8±10.8	521.4±11.5	550.3±11.8	540.9±6.5
PR, ms	47.0±1.5	45.8±1.1	42.9±2.6	44.5±1.2
QRS, ms	11.2±0.35	10.0±0.4	10.7±0.3	10.0±0.8
QTc, ms	26.0±1.3	24.1±1.0	30±1.5	28.7±2.4
SNRT, ms	154.1±4.0	155.1±8.9	143±5.7	161±9.9
AVERP	50.4±1.3	49.4±1.8	49.3±0.7	47±2.1
AERP, ms	43.5±1.9	40.5±1.5	39.3±0.7	36.4±2.0

Values are expressed as mean ± SEM. RR, interval from R to R-peak; HR, heart rate; PR interval, onset of P wave to peak of R wave; QRS, beginning of Q wave to the point of the S wave returning to the isoelectric line; QTc, rate corrected QT interval; SNRT, sinus node recovery time; AERP, atrial effective refractory period; AVERP, atria ventricular effective refractory period. None of the parameters changed significantly following tetracaine administration.